

GROWTH AND MATURATION OF SMALL HEPATOCYTES ISOLATED FROM ADULT RAT LIVER

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Small hepatocytes existed in the supernatant following low-speed centrifugation of the cell suspension after collagenase liver perfusion. The cells proliferated for more than 2 months and formed colonies in the Dulbecco's modified Eagle's medium supplemented with 10 mM nicotinamide, 10% fetal bovine serum, 1 mM ascorbic 2-phosphate, and 10 ng/ml epidermal growth factor. One small cell finally proliferated to several hundred cells. In addition, some cells in the colonies were shown to differentiate into mature hepatocytes that had a large cytoplasm and sometimes two nuclei. The secretion of albumin in the medium by the hepatocytes increased with time in culture, and the cells possessed connexin 32 in their cell membrane and many peroxisomes. Thus, the small hepatocytes may be "committed progenitor cells" which can further differentiate into mature hepatocytes.

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We have reported that the proliferation of adult rat hepatocytes is observed in serum-free medium supplemented with 10 mM nicotinamide and 10 ng/ml EGF (1, 2). The proliferating cells are mainly mononucleate and form small-cell colonies surrounded by mature hepatocytes. Although these cells in focal colonies show a less differentiated appearance, they immunocytochemically and ultrastructurally possess hepatic characteristics (3). The small hepatocytes can proliferate for about 3 weeks and they suddenly die because the cells reach confluence. This finding suggested that the small hepatocytes might continue to

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Abbreviations used: EGF, epidermal growth factor; DMSO, dimethyl sulfoxide; Asc2P, ascorbic acid 2-phosphate; DMEM, Dulbecco's modified Eagles' medium; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; FBS, fetal bovine serum; CK, cytokeratin; PC, parenchymal cell; NPC, non-parenchymal cell; AFP, A-fetoprotein; FITC, fluorescein isothiocyanate.

proliferate if they could be individually isolated. In this report we show that the small hepatocytes existed in the supernatant following low-speed centrifugation of the cell suspension after collagenase liver perfusion and that each small hepatocyte continued to proliferate for more than 2 months. In addition, some cells in the colonies were shown to differentiate into mature hepatocytes.

Materials and Methods

Isolation of cells: Male Sprague-Dawley rats, weighing 300 to 450 g were used to isolate cells in livers by the two-step liver perfusion method of Seglen (4) with some modification (2). The cells were suspended in L-15 medium with 0.2% BSA, 20 mM HEPES, 0.5 μ g/ml insulin, 10^{-7} M dexamethasone and antibiotics. The suspension was centrifuged at 50 x g for 1 min. Pellets (parenchymal cell [PC] fraction) were used for isolating parenchymal cells. The supernatant (non-parenchymal cell [NPC] fraction) was used to isolate individual small hepatocytes. The procedure was carried out as Tatenio and Yoshizato reported (5) with some modification. The supernatant was collected and centrifuged at 50 x g for 1 min. After this procedure was repeated, the supernatant was centrifuged at 150 x g for 5 min, and the pellet was then resuspended in fresh medium. This procedure was carried out twice. The number of cells in the suspension, the size of which seemed to be larger than that of lymphocytes, was counted. Then, 9×10^5 cells were inoculated onto 35-mm culture dishes coated with rat tail collagen. The cells were placed in an air incubator at 37°C. Two to three hours later, the medium was changed to DMEM supplemented with 10% FBS, 10 mM nicotinamide, 1 mM Asc2P, 10 ng/ml EGF, 0.5 μ g/ml insulin, 10^{-7} M dexamethasone, and antibiotics. The cells were placed in a 5% CO₂/95% air incubator at 37°C and 1% DMSO was added to the medium from 4 days after plating. In the deletion experiment, the DMEM without each substance was added from 2 to 3 hrs after plating. The medium was replaced with fresh medium every other day.

Photographs of Cells: The same fields of cultured cells identified by needle marks were photographed each day using an Olympus phase-contrast microscope.

Cytochemical Examinations and Colony Counts: For immunocytochemistry, mouse anti-CK8 (Amersham), rabbit anti-rat albumin (Cappel), and rabbit anti-AFP (MBL) antibodies were used as primary antibodies. We used the ABC method, and diaminobenzidine was used as the substrate for peroxidase staining. For immunofluorescent staining of connexin 32, we used rabbit anti-connexin 32 antibody (a gift from Dr. M. Mori) and FITC-conjugated anti-rabbit IgG. For enzyme-cytochemistry of catalase in peroxisomes, the method of Furukawa et al. (6) was used.

To count the number of colonies, we used CK8-positivity for the identification of the hepatocytes in the colonies and regarded a cell cluster consisting of more than 8 CK8-positive cells as a colony. The dishes were fixed with cold absolute ethanol at day 10. The numbers of both the colonies strongly stained with CK8 and the cells in each colony were counted. We observed 30 fields per dish and 4 dishes per batch.

Determination of rat albumin secretion: Quantification of the secreted albumin was done by ELISA. We used the two-antibody sandwich method (7).

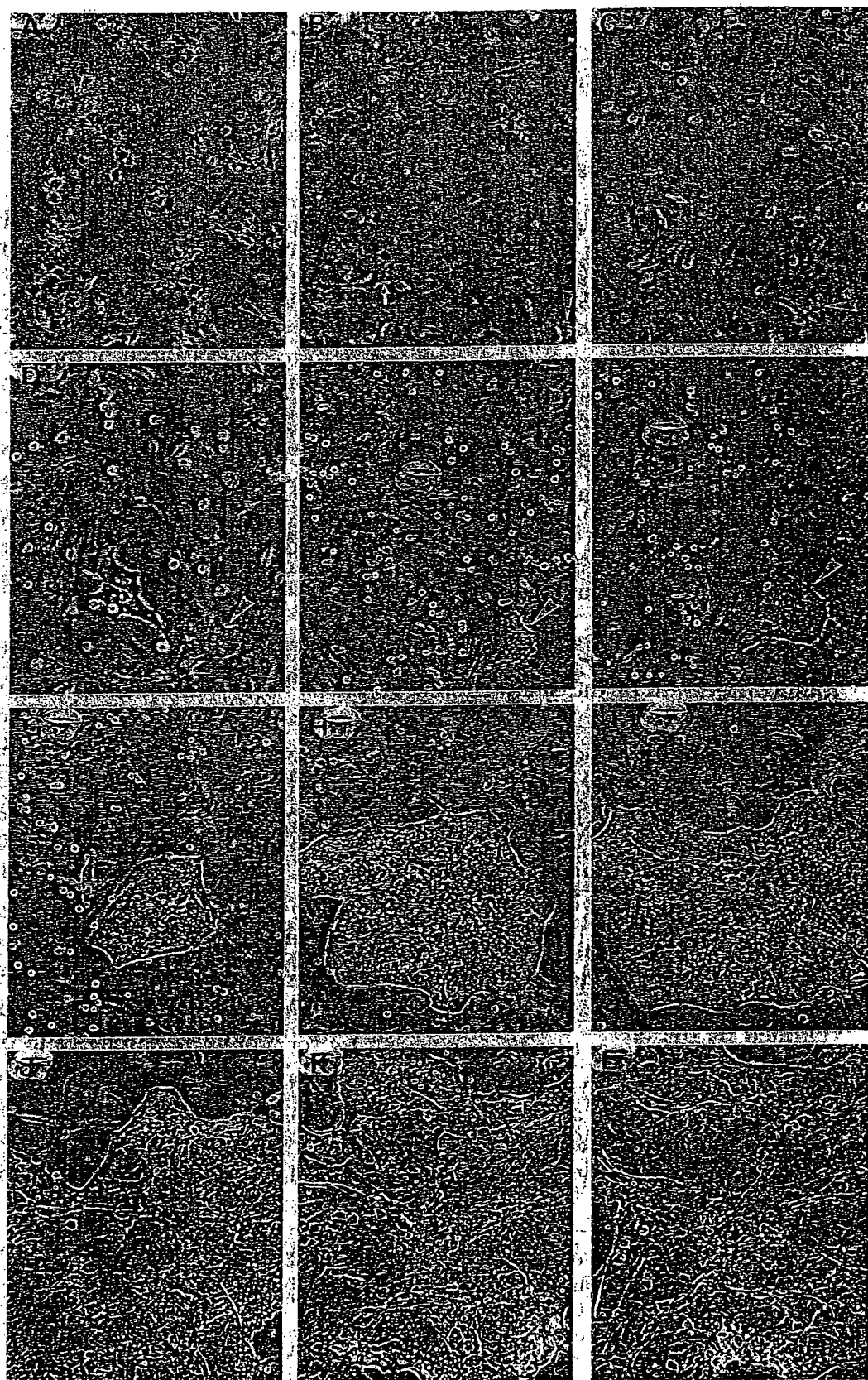
The primary antibody was goat anti-rat albumin antibody (Cappel). Peroxidase-conjugated rabbit anti-rat albumin antibody (Cappel) was subsequently used. ABTS was used as a chromogenic substrate.

Results and Discussion

Figure 1 shows that the isolated cells were of several types, which might be not only hepatocytes but also Ito cells, Kupffer cells, epithelial cells, endothelial cells, and so on. We could find some relatively large cells among these. The cells having bright nuclei and darkened cytoplasm seemed to be hepatocytes. Various cells, the size of which was one-third to one-half that of mature hepatocytes, existed among the hepatocytes. The cells began to divide from 3 days after plating and continued to proliferate for more than 2 months. Relatively large hepatocytes could proliferate but the number of divisions may be limited. One small cell finally proliferated to several hundred cells. About 20 days later, some cells in colonies possessed large cytoplasm and were binucleate. When a colony reached a certain size, it appeared to cease expanding. Such colonies may have reached equilibrium between the production of and the death of cells.

We examined what the most important substance was in the medium for the proliferation of the small cells (Fig. 2 and Table 1). Without 10 mM nicotinamide (Fig. 2B) or 10% FBS (Fig. 2C), few colonies were observed, the cells in the colonies consisted of relatively large cells, and the isolated cells seemed to adhere to each other. On the other hand, without 10 ng/ml EGF (Fig. 2D) or 1 mM Asc2P (Fig. 2E), although one-half to two-thirds of the number of the colonies were formed compared to the control, each colony consisted of a number of the cells similar to that of the control. Small hepatocytes in NPC fractions needed both nicotinamide and serum for their colony formation and their proliferation was independent of EGF. This result is contradictory to the previous one in that the small cells appeared in the serum-free medium and they required nicotinamide and EGF for their growth (2). However, this can be explained by the fact that EGF is necessary for mature hepatocytes to proliferate and to secrete plasma proteins such as albumin, transferrin and so on, and that the secreted proteins are substituted for the serum. Thus, the most important substance for the growth of small hepatocytes was nicotinamide. The 1% DMSO supplement did not affect the proliferation of hepatocytes but seemed to suppress

Figure 1. Phase-contrast photographs of cells cultured in DMEM supplemented with 10 mM nicotinamide, 10% FBS, 1 mM Asc2P, and 10 ng/ml EGF. 1% DMSO was added to the medium from day 4. The cells within the same field marked by a needle are shown. (A) day 1 (about 24 hr after plating), (B) day 3, (C) day 4, (D) day 6. An arrowhead shows a small hepatocyte forming a colony. An arrow shows a relatively large hepatocyte that has only divided twice. (A-D, $\times 70$) (E) day 10, (F) day 14, (G) day 18, (H) day 27, (I) day 31, (J) day 40, (K) day 52, (L) day 62. (E-L, $\times 48$)



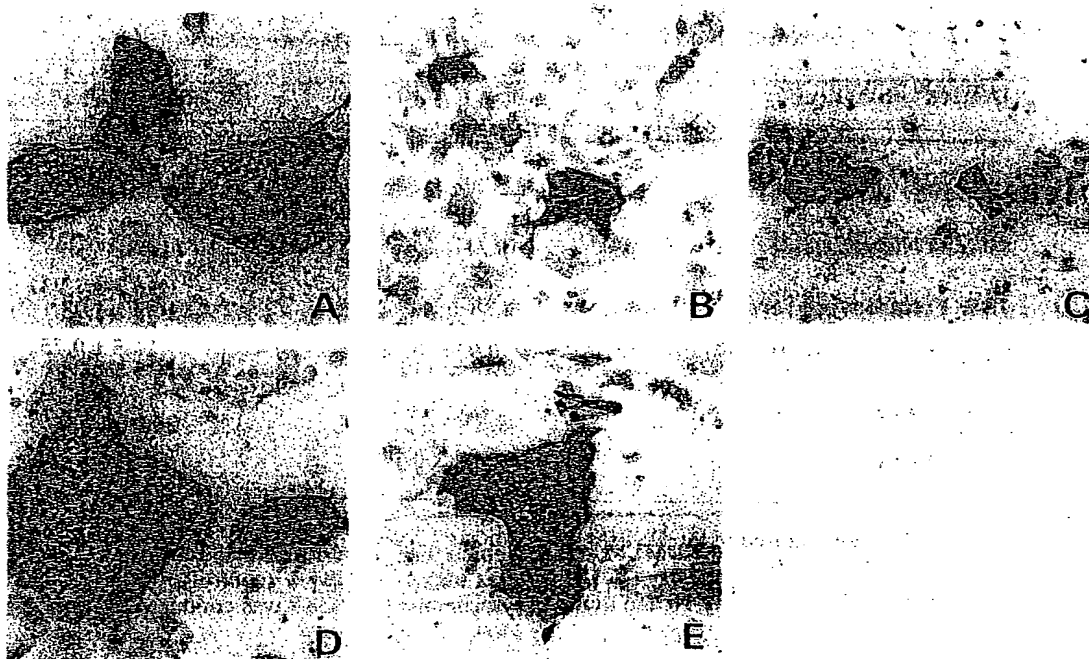


Figure 2. The formation of small-hepatocyte colonies in DMEM supplemented with 10 mM nicotinamide, 10% FBS, 1mM Asc2P, and 10 ng/ml EGF. 1% DMSO was added to the medium from day 4 (A; control). The cells were cultured in the medium without nicotinamide (B), FBS (C), Asc2P (D), or EGF (E). The dishes were fixed with cold absolute ethanol at day 10. Immunocytochemistry for CK8 was carried out for the identification of hepatocytes. Small hepatocytes in the colonies were strongly stained with CK8 (x83).

the growth of NP cells as we previously described (2). As shown in Fig. 3, the amount of albumin in the culture medium increased as the colonies developed with time in culture. To examine whether the cells in the colonies were truly hepatocytes, cytochemical stainings were carried out as it is known that hepatocytes must possess albumin and peroxisomes in their cytoplasm and connexin 32 in the cell membrane (Fig. 4). The cells in the colonies showed positivity to albumin and connexin 32. In addition, many cells in the colonies had numerous large peroxisomes in their cytoplasm. Ultrastructurally, peroxisomes with a crystalline nucleoid were often observed in the cells (data not shown). Some hepatocytes in each colony randomly expressed AFP, but no relationship was observed between the AFP-positivity and the size of the cells.

In the present experiment we showed that the proliferating hepatocytes existed in the NPC fractions of a cell suspension after collagenase perfusion of adult liver. The size of the cells was smaller than that of mature hepatocytes. However, their appearance was that of true hepatocytes and they had hepatic characteristics as shown by immunocytochemistry. It is of interest that some cells in colonies became mature hepatocytes which possessed a large cytoplasm and sometimes two nuclei. In addition, the other cells in the colony still maintained the ability to proliferate. Furthermore, relatively large hepatocytes

Table 1
Number of colonies and of small hepatocytes per colony

Deleted substances	No. of Colonies per square millimeter	No. of Cells per Colony
Control	2.13 ± 0.12	26.73 ± 3.8
10 mM Nicotinamide	0.31 ± 0.1	11.93 ± 2.49
10% Fetal Bovine Serum	0.38 ± 0.09	16.3 ± 1.93
1 mM Ascorbic 2-Phosphate	0.93 ± 0.12	16.5 ± 0.75
10 ng/ml EGF	1.37 ± 0.2	20.7 ± 3.16
1% DMSO	1.97 ± 0.17	27.01 ± 7.3

The cells were cultured in the DMEM supplemented with 10 mM nicotinamide, 10% FBS, 1mM Asc2P, and 10 ng/ml EGF. 1% DMSO was added from day 4 (control). The dishes were fixed at day 10 and immunocytochemistry for CK8 was conducted.

in the cell suspension could also attach on the dish and divide several times, but they could not form a colony. As we have proposed, hepatocytes may be classified into three types of cells with respect to their ability to divide (8-10): (a) cells that have a high potential to proliferate and form colonies (Type I cells); (b) cells for which the number of possible cell divisions is limited to several divisions (Type II cells); (c) cells that lose the ability to divide (Type III cells). The small hepatocytes shown in this experiment seemed to be Type I cells, and might be "committed progenitor cells" which can further differentiate into mature hepatocytes. No clear marker was found to distinguish the cells from

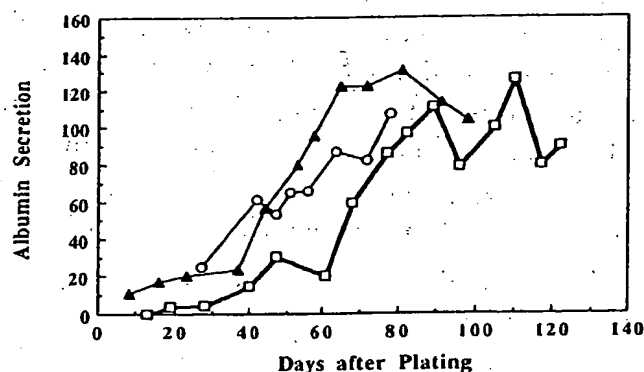


Figure 3. Measurement of rat albumin in the medium secreted by small hepatocytes. The cells were cultured in the medium supplemented with 10 mM nicotinamide; 10% FBS, 1mM Asc2P, and 10 ng/ml EGF. 1% DMSO was added to the medium from day 4. The medium was collected at the time of medium changes from the same dishes. An average of two dishes per experiment is represented in the figure and 3 separate experiments were carried out. The ordinate indicates the amount of albumin in the medium ($\mu\text{g/ml/48 hr}$). The abscissa shows the days after plating.

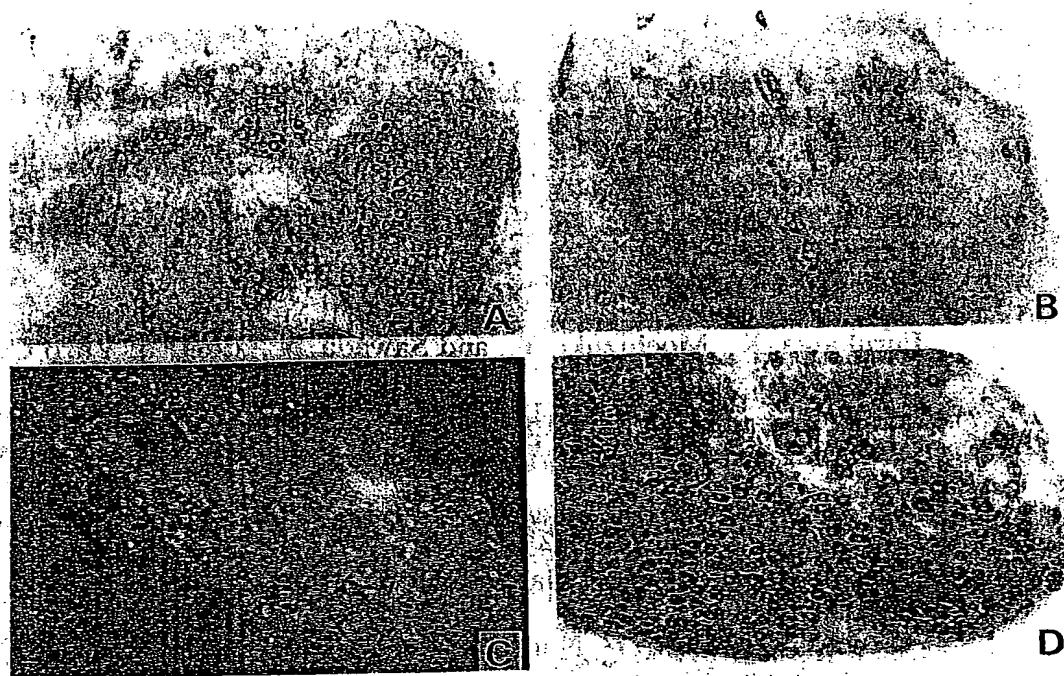


Figure 4. Cytochemical studies of small-hepatocytes in the colonies. (A) Immunocytochemistry for rat albumin at day 43. Most cells were positively stained (x83). (B) Immunocytochemistry for rat AFP at day 50. Some cells in the colony were randomly stained (x83). (C) Immunofluorescence for connexin 32 at day 36. To induce connexin 32, 2% DMSO, 10^{-5} M dexamethasone and 10^{-7} M glucagon were added to the medium for 10 days (x165). (D) Enzyme-cytochemistry for catalase in peroxisomes at day 115. Relatively large cells have many granules in their cytoplasm, which is darkened (x83).

other types of cells. Until now, the size of cells has been considered to be the most important factor in how many times hepatocytes divide. Barrandon and Green reported that clonogenicity of human keratinocytes in epidermis was closely related to cell size (11). The smallest cells were the most clonogenic. Although we still have to investigate where they are in the hepatic lobule, the existence of small hepatocytes in both PC and NPC fractions suggest that they may be inserted hepatic plates in the lobule and may be mistaken for mature hepatocytes.

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